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(54) Title: BLEACHING OF FABRIC WITH A HYBRID ENZYME CONTAINING A PHENOL OXIDIZING ENZYME FUSED TO A CELLULOSE BINDING DOMAIN

(57) Abstract

A process for providing a bleached look in the colour density of the surface of dyed fabric, the process comprising contacting, in an aqueous medium, a dyed fabric with a hybrid enzyme comprising a phenol oxidizing enzyme fused to an amino acid sequence having a cellulose-binding domain, and a hydrogen peroxide source when the phenol oxidizing enzyme is a peroxidase.

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BLEACHING OF FABRIC WITH A HYBRID ENZYME CONTAINING A PHENOL OXIDIZING ENZYME FUSED TO A CELLULOSE BINDING DOMAIN

FIELD OF INVENTION

The present invention relates to a process for providing a bleached look in the colour density of the surface of dyed fabric, especially cellulosic fabric such as denim.

BACKGROUND ART

The most usual method of providing a bleached stone-washed look in denim fabric or jeans is by washing the denim or jeans made from such fabric in the presence of pumice stones to provide the desired localized lightening of the colour of the fabric. This is then followed by a bleaching process where the fabric is treated with sodium hypochlorite typically at 60°C and pH 11-12 for up to 20 min., followed by a neutralisation step and a rinsing. Use of hypochlorite is undesirable, both because chlorite itself is undesirable and because the neutralisation subsequently generates high amounts of salts leading to disposal and pollution problems.

Bleaching enzymes such as peroxidases together with hydrogen peroxide or oxidases together with oxygen have also been suggested for bleaching of dyed textiles (see WO 92/18683), either alone or together with a phenol such as p-hydroxycinnamic acid, 2,4-dichlorophenol, p-hydroxybenzene sulphonate, vanillin or p-hydroxybenzoic acid. However the disclosed process is not efficient.

Thus there is still a need for providing a process which is environmentally friendly and which gives the desired bleached look in dyed fabrics. The problem to be solved is not easy as many VAT-dyes, especially indigo, are not soluble in water and have a very compact structure on the fibre surface, making them difficult for an enzyme to attack.

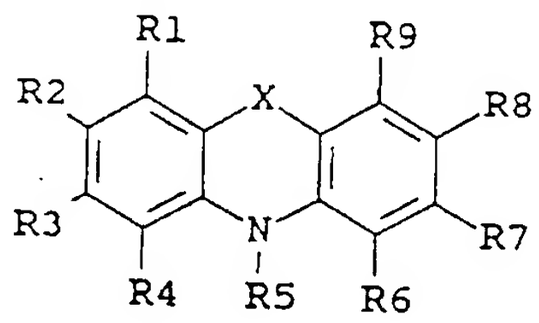
SUMMARY OF THE INVENTION

Surprisingly it has been found that it is possible to create a very efficient process for providing a bleached look in the colour density of the surface of dyed fabric, the process comprising contacting, in an aqueous medium, a dyed fabric with

a) a hybrid enzyme comprising a phenol oxidizing enzyme fused to an amino acid sequence having a cellulose-binding domain, and

b) a hydrogen peroxide source when the phenol oxidizing enzyme is a peroxidase.

Additionally, it may in some cases be an advantage also to add an enhancing agent of the following formula:

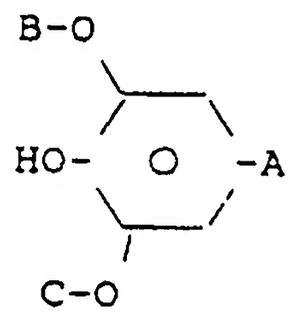


in which formula X represents (-O-) or (-S-), and the substituent groups R^1-R^9, which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, aryl-C_1-C_5-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group R^{10}; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups R^{10}; and which C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, and aryl-C_1-C_5-alkyl groups may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups R^{10};

which substituent group R^{10} represents any of the following radicals: halogen, hydroxy, formyl, carboxy and

esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidin-1-yl, C_1-C_5-alkyl, C_1-C_5-alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy, C_1-C_5-alkyl, C_1-C_5-alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which C_1-C_5-alkyl, and C_1-C_5-alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl;

or in which general formula two of the substituent groups R^1-R^9 may together form a group -B-, in which B represents any of the following the groups: ($-CHR^{10}-N=N-$), $(-CH=CH-)_n$, $(-CH=N-)_n$ or ($-N=CR^{10}-NR^{11}-$), in which groups n-represents an integer of from 1 to 3, R^{10} is a substituent group as defined above and R^{11} is defined as R^{10}. (It is to be understood that if the above mentioned formula comprises two or more R^{10}-substituent groups, these R^{10}-substituent groups may be the same or different); or an enhancing agent of the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, $-SO_2$-E, -N-XY, and $-N^+$-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C_1-C_{16} alkyl, preferably a C_1-C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and

optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; $1 \leq m \leq 5$.

DETAILED DESCRIPTION OF THE INVENTION

Dyed Fabric

The process of the invention is most beneficially applied to cellulose-containing fabrics, such as cotton, viscose, rayon, ramie, linen, Tencel, or mixtures thereof, or mixtures of any of these fibres, or mixtures of any of these fibres together with synthetic fibres such as mixtures of cotton and spandex (stretch-denim). In particular, the fabric is denim.

The fabric may be dyed with vat dyes such as indigo, or indigo-related dyes such as thioindigo.

In a most preferred embodiment of the process of the invention, the fabric is indigo-dyed denim, including clothing items manufactured therefrom.

Enzyme hybrids

According to the invention an enzyme hybrid comprises a phenol oxidizing enzyme fused to an amino acid sequence having a cellulose-binding domain.

By the term "a phenol oxidizing enzyme" is meant an enzyme, which by using hydrogen peroxide or molecular oxygen, is capable of oxidizing organic compounds containing phenolic groups. Examples of such enzymes are peroxidases and oxidases.

Enzyme hybrids are known in the art, (for reference see e.g. WO 90/00609 and WO 95/16782): They may be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest and growing the host cell to express the fused gene. The enzyme hybrids may be described by the following formula:

CBD - MR - X,

wherein:

CBD can be either the N-terminal or the C-terminal region of an amino acid sequence corresponding to at least the cellulose-binding domain;

MR is the middle region (the linker), and may be a bond, or a short linking group of from about 2 to about 100 carbon atoms, in particular of from 2 to 40 carbon atoms, or typically from about 2 to about 100 amino acids, in particular of from 2 to 40 amino acids, and

X can be either the N-terminal or the C-terminal region and is a phenol oxidizing enzyme.

Cellulose-Binding Domains

The term "cellulose-binding domain" is intended to be understood as defined by Peter Tomme et al. "Cellulose-Binding Domains: Classification and Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996.

This definition classifies more than 120 cellulose-binding domains (CBDs) into 10 families (I-X), and it demonstrates that CBDs are found in various enzymes such as cellulases, xylanases, mannanases, arabinofuranosidases, acetyl esterases and chitinases. CBDs have also been found in algae, e.g., the red alga Porphyra purpurea as a non-hydrolytic polysaccharide-binding protein, for reference see Peter Tomme et al., supra.

However, most of the CBDs are from cellulases and xylanases. CBDs are found at the N or C termini of proteins or are internal.

Cellulases useful for preparation of Cellulose-Binding Domains

The techniques used in isolating a cellulase gene are well known in the art.

In the present context, the term "cellulase" refers to an enzyme which catalyses the degradation of cellulose to glucose, cellobiose, triose and other cello-oligosaccharides.

In a preferred embodiment of the invention, the cellulase to be used in the method is an endoglucanase (EC

3.2.1.4), preferably a monocomponent (recombinant) endoglucanase.

Preferably, the cellulase is a microbial cellulase, more preferably a bacterial or fungal cellulase.

Useful examples of bacterial cellulases are cellulases derived from or producible by bacteria from the group consisting of Pseudomonas, Bacillus, Cellulomonas, Clostridium, Microspora, Thermotoga, Caldocellum and Actinomycets such as Streptomyces, Termomonospora and Acidothemus, in particular from the group consisting of Pseudomonas cellulolyticus, Bacillus lautus, Cellulomonas fimi, Microspora bispora, Termomonospora fusca, Termomonospora cellulolyticum and Acidothemus cellulolyticus.

The cellulase may be an acid, a neutral or an alkaline cellulase, i.e. exhibiting maximum cellulolytic activity in the acid, neutral or alkaline range, respectively.

A useful cellulase is an acid cellulase, preferably a fungal acid cellulase, which is derived from or producible by fungi from the group of genera consisting of Trichoderma, Myrothecium, Aspergillus, Phanaerochaete, Neurospora, Neocallimastix and Botrytis.

A preferred useful acid cellulase is derived from or producible by fungi from the group of species consisting of Trichoderma viride, Trichoderma reesei, Trichoderma longibrachiatum, Myrothecium verrucaria, Aspergillus niger, Aspergillus oryzae, Phanaerochaete chrysosporium, Neurospora crassa, Neocallimastix partriciarum and Botrytis cinerea.

Another useful cellulase is a neutral or alkaline cellulase, preferably a fungal neutral or a fungal alkaline cellulase, which is derived from or producible by fungi from the group of genera consisting of Aspergillus, Penicillium, Myceliophthora, Humicola, Irpex, Fusarium, Stachybotrys, Scopulariopsis, Chaetomium, Mycogone, Verticillium, Myrothecium, Papulospora, Gliocladium, Cephalosporium and Acremonium.

A preferred alkaline cellulase is derived from or producible by fungi from the group of species consisting of Humicola insolens, Fusarium oxysporum, Myceliopthora thermophila, Penicillium janthinellum and Cephalosporium sp.,

preferably from the group of species consisting of Humicola insolens, DSM 1800, Fusarium oxysporum, DSM 2672, Myceliophthora thermophila, CBS 117.65, and Cephalosporium sp., RYM-202.

A preferred example of a native or parent cellulase is an alkaline endoglucanase which is immunologically reactive with an antibody raised against a highly purified ≅43kD endoglucanase derived from Humicola insolens, DSM 1800, or which is a derivative of the ≅43kD endoglucanase exhibiting cellulase activity.

Other examples of useful cellulases are variants having, as a parent cellulase, a cellulase of fungal or bacterial origin, e.g. a cellulase derivable from a strain of the fungal genus Humicola, Trichoderma or Fusarium.

The techniques used in isolating a xylanase gene, a mannanase gene, an arabinofuranosidase gene, an acetyl esterase gene or a chitinase gene are also well known in the art.

Isolation of a Cellulose-Binding domain

In order to isolate the cellulose binding domain of e.g. a cellulase, several genetic approaches may be used. One method uses restriction enzymes to remove a portion of the gene and then to fuse the remaining gene-vector fragment in frame to obtain a mutated gene that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as Ba131 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene deletion methods result in a mutated gene encoding a shortened gene molecule which may then be evaluated for substrate binding ability. Appropriate substrates for evaluating the binding activity include compounds such as Avicel and cotton fibres.

Once a nucleotide sequence encoding the substrate binding region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding the enzyme of interest. The cellulose binding encoding fragment and the DNA

encoding the enzyme of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to provide for expression. Microbial hosts such as Aspergillus, e.g., A. niger and A. oryzae, Bacillus, E. coli or S.cerevisiae are preferred.

Peroxidases

Suitable peroxidases to be fused with the sequence encoding the cellulose-binding domain may be any peroxidase enzyme comprised by the enzyme classification (EC 1.11.1.7), or any fragment derived therefrom, exhibiting peroxidase activity. Preferably, the peroxidase employed in the method of the invention is producible by plants (e.g. horseradish or soybean peroxidase) or microorganisms such as fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g., Fusarium, Humicola, Tricoderma, Myrothecium, Verticillum, Arthromyces, Caldariomyces, Ulocladium, Embellisia, Cladosporium or Dreschlera, in particular Fusarium oxysporum (DSM 2672), Humicola insolens, Trichoderma resii, Myrothecium verrucana (IFO 6113), Verticillum alboatrum, Verticillum dahlie, Arthromyces ramosus (FERM P-7754), Caldariomyces fumago, Ulocladium chartarum, Embellisia alli or Dreschlera halodes.

Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. Coprinus, Phanerochaete, Coriolus or Trametes, in particular Coprinus cinereus f. microsporus (IFO 8371), Coprinus macrorhizus, Phanerochaete chrysosporium (e.g. NA-12) or Trametes (previously called Polyporus), e.g. T. versicolor (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. Rhizopus or Mucor, in particular Mucor hiemalis.

Some preferred bacteria include strains of the order Actinomycetales, e.g., Streptomyces spheroides (ATTC 23965), Streptomyces thermoviolaceus (IFO 12382) or Streptoverticillum verticillium ssp. verticillium.

Other preferred bacteria include Bacillus pumilus (ATCC 12905), Bacillus stearothermophilus, Rhodobacter sphaeroides, Rhodomonas palustri, Streptococcus lactis, Pseudomonas purrocinia (ATCC 15958) or Pseudomonas fluorescens (NRRL B-11).

Further preferred bacteria include strains belonging to Myxococcus, e.g., M. virescens.

Particularly, a recombinantly produced peroxidase is preferred, e.g., a peroxidase derived from a Coprinus sp., in particular C. macrorhizus or C. cinereus according to WO 92/16634, or a variant thereof, e.g., a variant as described in WO 94/12621.

Laccases and Laccase Related Enzymes

Suitable laccases to be fused with the sequence encoding the cellulose-binding domain include laccases and laccase related enzymes, i.e., any laccase enzyme comprised by the enzyme classification (EC 1.10.3.2), any chatechol oxidase enzyme comprised by the enzyme classification (EC 1.10.3.1), any bilirubin oxidase enzyme comprised by the enzyme classification (EC 1.3.3.5) or any monophenol monooxygenase enzyme comprised by the enzyme classification (EC 1.14.18.1).

The laccase enzymes are known from microbial and plant origin. The microbial laccase enzyme may be derived from bacteria or fungi (including filamentous fungi and yeasts) and suitable examples include a laccase derivable from a strain of Aspergillus, Neurospora, e.g., N. crassa, Podospora, Botrytis, Collybia, Fomes, Lentinus, Pleurotus, Trametes, e.g., T. villosa and T. versicolor, Rhizoctonia, e.g., R. solani, Coprinus, e.g. C. plicatilis and C. cinereus, Psatyrella, Myceliophthora, e.g. M. thermophila, Scytalidium, Polyporus, e.g., P. pinsitus, Phlebia, e.g., P. radita (WO 92/01046), or Coriolus, e.g., C. hirsutus (JP 2-238885), in particular laccases obtainable from Trametes, Myceliophthora, Scytalidium or Polyporus.

Plasmids

Preparation of plasmids capable of expressing fusion proteins having the amino acid sequences derived from fragments of more than one polypeptide are well known in the art, for reference see e.g. WO 90/00609. The expression cassette may be included within a replication system for episomal maintenance in an appropiate cellular host or may be provided without a replication system, where it may become integrated into the host genome. The DNA may be introduced into the host in accordance with known techniques such as transformation, microinjection or the like.

Once the fused gene has been introduced into the appropiate host, the host may be grown to express the fused gene. Normally it is desirable additionally to add a signal sequence which provides for secretion of the fused gene.

Typical examples of useful fused genes are:

Signal sequence -- (pro-peptide) -- Cellulose-Binding Domain -- Linker -- Enzyme of interest, or

Signal sequence -- (pro-peptide) -- Enzyme of interest -- Linker -- Cellulose-Binding Domain, in which the pro-peptide normally contains 5-25 amino acids.

The recombinant product may be glycosylated or non-glycosylated.

Phenol Oxidizing Enzyme Systems

If the phenol oxidizing enzyme requires a source of hydrogen peroxide, the source may be hydrogen peroxide or a hydrogen peroxide precursor for in situ production of hydrogen peroxide, e.g., percarbonate or perborate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and a substrate for the oxidase, or an amino acid oxidase and a suitable amino acid, or a peroxycarboxylic acid or a salt thereof. Hydrogen peroxide may be added at the beginning of or during the process, e.g. in a concentration corresponding to 0.001-25 mM H_2O_2.

If the phenol oxidizing enzyme requires molecular oxygen, molecular oxygen from the atmosphere will usually be present in sufficient quantity.

According to the invention the concentration of the hybrid enzyme in the aqueous medium where the localized variation in the colour density of the surface of the dyed fabric is taking place, may be 0.001-10000 µg of hybrid enzyme protein per g of fabric, preferably 0.1-1000 µg of hybrid enzyme protein per g of fabric, more preferably 1-100 µg of hybrid enzyme protein per g of fabric.

If the phenol oxidizing enzyme is a peroxidase, the peroxidase activity may be determined in the following way:

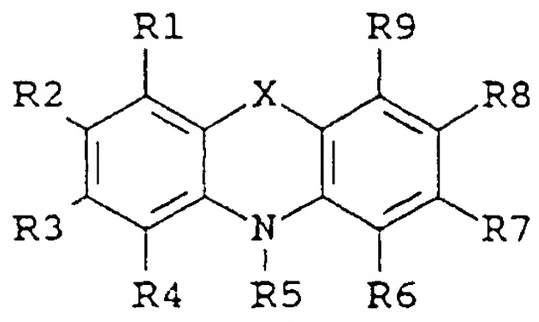
1 peroxidase unit (PODU) is the amount of enzyme that catalyzes the conversion of 1 µmole hydrogen peroxide per minute at the following analytical conditions: 0.88 mM hydrogen peroxide, 1.67 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), 0.1 M phosphate buffer, pH 7.0, incubated at 30°C, photometrically followed at 418 nm.

If the phenol oxidizing enzyme is a laccase, the laccase activity may be determined in the following way:

Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 µM syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time. 1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 µmole syringaldazin per minute at these conditions.

Enhancing Agents

The enhancing agent used in the present invention may be described by the following formula:



in which formula X represents (-O-) or (-S-), and the substituent groups R^1-R^9, which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, aryl-C_1-C_5-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group R^{10}; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups R^{10}; and which C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, and aryl-C_1-C_5-alkyl groups may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups R^{10};

which substituent group R^{10} represents any of the following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidin-1-yl, C_1-C_5-alkyl, C_1-C_5-alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy, C_1-C_5-alkyl, C_1-C_5-alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which C_1-C_5-alkyl, and C_1-C_5-alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl,

sulfo and esters and salts hereof, and sulfamoyl;

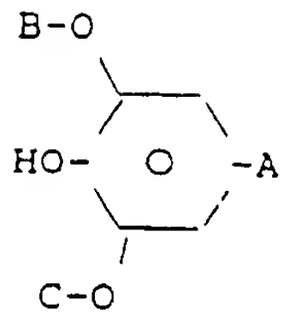
or in which general formula two of the substituent groups R^1-R^9 may together form a group -B-, in which B represents any of the following the groups: $(-CHR^{10}-N=N-)$, $(-CH=CH-)_n$, $(-CH=N-)_n$ or $(-N=CR^{10}-NR^{11}-)$, in which groups n-represents an integer of from 1 to 3, R^{10} is a substituent group as defined above and R^{11} is defined as R^{10}. (It is to be understood that if the above mentioned formula comprises two or more R^{10}-substituent groups, these R^{10}-substituent groups may be the same or different).

In particular embodiments, the enhancing agent is 10-methylphenothiazine, phenothiazine-10-propionic acid, N-hydroxysuccinimide phenothiazine-10-propionate, 10-ethyl-phenothiazine-4-carboxylic acid, 10-ethylphenothiazine, 10-propylphenothiazine, 10-isopropylphenothiazine, methyl phenothiazine-10-propionate, 10-phenylphenothiazine, 10-allylphenothiazine, 10-(3-(4-methylpiperazin-1-yl)propyl)phenothiazine, 10-(2-pyrrolidin-1-yl-ethyl)phenothiazine, 2-methoxy-10-methyl-phenothiazine, 1-methoxy-10-methylphenothiazine, 3-methoxy-10-methylphenothiazine, 3,10-dimethylphenothiazine, 3,7,10-trimethylphenothiazine, 10-(2-hydroxyethyl)phenothiazine, 10-(3-hydroxypropyl)phenothiazine, 3-(2-hydroxyethyl)-10-methylphenothiazine, 3-hydroxymethyl-10-methylphenothiazine, 3,7-dibromophenothiazine-10-propionic acid, phenothiazine-10-propionamide, chlorpromazine, 2-chloro-10-methylphenothiazine, 2-acetyl-10-methylphenothiazine, 10-methylphenoxazine, 10-ethylphenoxazine, phenoxazine-10-propionic acid, 10-(2-hydroxyethyl)phenoxazine or 4-carboxyphenoxazine-10-propionic acid.

The enhancing agent of the invention may be present in concentrations of from 0.005 to 1000 µmole per g of fabric, preferably 0.05 to 100 µmole per g of fabric, more preferably 0.05 to 10 µmole per g of fabric.

Enhancing Agents

The enhancing agent used in the present invention may be described by the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, $-SO_2$-E, -N-XY, and $-N^+$-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C_1-C_{16} alkyl, preferably a C_1-C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; $1 \leq m \leq 5$.

In a preferred embodiment A in the above mentioned formula is -CO-E, in which E may be -H, -OH, -R, or -OR; R being a C_1-C_{16} alkyl, preferably a C_1-C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; $1 \leq m \leq 5$.

In the above mentioned formula A may be placed meta to the hydroxy group instead of being placed in the para-position as shown.

In particular embodiments, the enhancing agent is acetosyringone, methylsyringate, ethylsyringate, propyl-syringate, butylsyringate, hexylsyringate, or octylsyringate.

The enhancing agent of the invention may be present in concentrations of from 0.005-1000 µmole per g of fabric, preferably 0.05-100 µmole per g of fabric, more preferably 0.05-10 µmole per g of fabric.

Industrial Applications

The process of the present invention is typically used in industrial machines for making fabric look bleached.

Normally, the process of the invention will be

performed on fabric already stonewashed, but the process may also be applied to fabric which has not undergone a stonewashing process beforehand. Most commonly the fabric is added to the machine according to the machine capacity per the manufacturer's instructions. The fabric may be added to the machine prior to introducing water or the fabric may be added after water is introduced. The hybrid enzyme, the enhancing agent, and in some cases the hydrogen peroxide source may be present in the water prior to adding the fabric or they may be added after the fabric has been wetted. The hybrid enzyme may be added simultaneously with the enhancing agent or they may be added separately. After the fabric has been contacted with the hybrid enzyme and the enhancing agent it should be agitated in the machine for a sufficient period of time to ensure that the fabric is fully wetted and to ensure the action of the enzyme system and the enhancing agent.

The optimum bleaching conditions may be a compromise between optimum stability of the enzyme, optimum activity of the enzyme, optimum stability of the radical of the enhancing agent, and optimum reactivity (oxidation potential) of the radical, as well as choice of buffering system (buffer capacity, buffer toxicity, costs of buffer etc.).

Test Procedure

The test procedure for fabric bleaching, in particular denim bleaching, may be performed as described below, or as described in Examples 2 and 3:

Procedure: 18 ml 0.01 M B&R (Britt & Robinson) buffer (pH 4, 6, or 8) are added to a 50 ml conical flask. A magnet bar (4 cm) and a circular piece of stone washed denim (3.5 cm diameter ≅ 0.4 g) are added to the flask together with 1 ml of enzyme and optionally 1 ml of the stock solution of an enhancing agent, giving a denim:liquor (w/w) ratio of 1:50. The flask is incubated for 2-3 hours on a magnet stirrer in a water bath (50°C and approximately 200 rpm). After the enzymatic bleaching, the denim swatch is rinsed with distilled water and air dried, whereafter it is evaluated for the degree

of bleaching. The evaluation is performed visually and by using a Minolta Chroma Meter CR200 or a Minolta Chroma Meter CR300.

Evaluation: A Minolta Chroma Meter CR200 or CR300 (available from Minolta Corp.) is used according to Manufacturer's instructions to evaluate the degree of bleaching as well as to estimate any discoloration using the change in the colour space coordinates L*a*b* (CIELAB-system): L* gives the change in white/black at a scale of from 0 to 100, a* gives the change in green (-a*)/red (+a*), and b* gives the change in blue (-b*)/yellow (+b*). A decrease in L* means an increase in black colour (decrease of white colour), an increase in L* means an increase in white colour (a decrease in black colour), a decrease in a* means an increase in green colour (decrease in red colour), an increase in a* means an increase in red colour (a decrease in green colour), a decrease in b* means an increase in blue colour (a decrease in yellow colour), and an increase in b* means an increase in yellow colour (a decrease in blue colour).

The bleached stone washed denim swatches are compared to non-treated stone washed denim swatches.

The Minolta Chroma Meter CR200 or the Minolta Chroma Meter CR300 is operated in the L*a*b* colour space (coordinate system). The light source used is a CIE light standard C. Each measurement is an average of 3 measurements. The instrument is calibrated using a Minolta calibration plate (white). 10 non-treated denim swatches are measured 2 times each and the average of the coordinates L*a*b* are calculated and entered as a reference. The coordinates of the samples are then calculated as the difference (Δ) of the average of 3 measurements on each swatch from the reference value of the coordinates L*a*b*.

Visually a ΔL^* around 5 gives a significant effect.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1

Construction of a peroxidase fused to a cellulose binding domain (CiP-CBD and mCiP842-CBD)

This example concerns fusion proteins comprising a CBD linked to *Coprinus cinereus* peroxidase (CiP) or to a mutant thereof (mCiP842) (see, e.g., WO 95/10602).

Yeast expression system

The pJC106/YNG344 host/vector system was chosen as the standard expression system for all CiP experiments utilizing yeast expression. Mutant mCiP842 contains the following amino acid substitutions relative to the parent CiP: V53A, E239G, M242I, Y272F.

Construction of the CBD-CiP fusion vector JC20A or JC20D: CiP signal seq.-*H. insolens* family 45 cellulase CBD-*H. insolens* family 45 cellulase linker-CiP, or CiP signal seq.-*H. insolens* family 45 cellulase CBD-*H. insolens* family 45 cellulase linker-mCiP842.

The CBD-CiP fusion was constructed by amplifying four separate gene fragments using PCR. A) The CiP 5'-untranslated region and the CiP coding sequence from plasmid JC106 or mCiP842 encoding amino acids 1 to 22, B) the *H. insolens* family 45 cellulase CBD from plasmid pCaHj418 encoding amino acids 248-305, C) the *H. insolens* family 45 cellulase linker domain from plasmid pCaHj418 encoding amino acids 213-247, and D) the CiP coding sequence from plasmid JC106 or mCiP842 encoding amino acids 21 to 344.

The sequence of the *H. insolens* family 45 cellulase is disclosed in WO 91/17244.

Primers used in amplifications A through D were as follows:

Amplification A:

1. CiPpcrdwn: CCCCCTTCCCTGGCGAATTCCGCATGAGG
2. JC20.1: ACCTTGGGGTAGAGCGAGGGCACCGATG

Amplification B:

3. JC20.2: TGCACTGCTGAGAGGTGGGC

4. JC20.3: CAGGCACTGATGATACCAGT

Amplification C:

5. JC20.4: CCCTCCAGCAGCACCAGCTCT

6. JC20.5: TCCTCCAGGACCCTGACCGCTCGGAGTCGTAGGCTG

Amplification D:

7. JC20.6: TACGACTCCGAGCGGTCAGGGTCCTGGAGGAGGCGGG

8. YES2term: GGGAGGGCGTGAATGTAAG

Amplified products of reactions A) and B) were purified and phosphorylated using T4 polynucleotide kinase, ligated to one another for 15 min. at room temperature, and amplified with primers 1 and 4 to generate product AB. Amplified products of reactions C) and D) were purified and mixed, then PCR-amplified to generate product CD. Reaction products AB and CD were purified and phosphorylated using T4 polynucleotide kinase, ligated to one another for 15 min. at room temperature, and amplified with primers 1 and 8 to generate the final product. The resulting product was purified, mixed with plasmid JC106 which had the CiP gene removed by digestion with BamHI and XhoI. Plasmid JC20A contains the wild type CiP gene, whereas plasmid JC20D contains the peroxide-stable mutant mCiP842. Transformants were selected on minimal media lacking uridine.

Scoring of transformants for peroxidase and cellulose-binding activity

Plate Assay: Yeast transformants were grown on minimal media plates containing 2% galactose (to induce the GAL1 yeast promoter driving CBD-CiP expression) that had been covered with a double filter layer consisting of cellulose acetate on top of nitrocellulose. After overnight growth, both filters were washed twice with 100 ml of 20 mM phosphate buffer, pH 7.0 for 5 minutes, after which no colony debris could be detected. Filters were then assayed for bound peroxidase activity by coating them with a 100 mM phosphate buffer, pH 7.0, containing 50 μg/ml of diamino-benzidine and

1 mM hydrogen peroxide. Bound peroxidase activity appears as a brown precipitate on the filter.

Liquid Assay: Liquid cultures of mutants demonstrating cellulose binding in the filter assay were grown overnight in minimal media containing 2% galactose. 20 µl samples of culture broth were mixed with Avicel crystalline cellulose (20 g/L) in 0.1 M phosphate buffer, pH 7, 0.01% Tween 20 in a total volume of 100 µl and incubated at 22°C for 10 minutes. The mixture was then centrifuged to pellet the insoluble cellulose fraction, and the supernatants were assayed for peroxidase activity. Binding was scored as the % activity bound to the insoluble cellulose fraction based on the decrease in soluble activity.

High pH/thermal stability screening of CBD-CiP fusions

This screening process utilizes broth samples from yeast cultures grown in microtiter plates. The 96-well plate screen is performed by first growing yeast transformants of a pool of mutants in 50 µL volumes of URA(-) medium, pH 6.0 in 96-well microtiter plates. Cultures are inoculated by dilution into medium and pipetting (robotic or manual autopipettor) into 96-well plates. These are placed in an incubator set at 30°C, 350 RPM and shaken for approximately 5 days. Plates are placed directly from the culture box onto the robotic system.

Both CiP and mCiP842 and the related fusion proteins were subjected to a combined pH - temperature - H_2O_2 stress test: After an initial activity assay, cultures are diluted to ca. 0.06 PODU/ml, and incubated in 200 µM hydrogen peroxide, 100 mM phosphate/borate buffer, pH 10.5 at 50°C. After 0, 10, 20 and 30 minutes, samples are removed and residual activity is measured using the standard ABTS assay, pH 7.0. Improved mutants are those showing higher residual activity than CiP and are expressed as percent residual activity relative to the time 0 assay result.

Yeast expression plasmids designed to make *H. insolens* family 45 cellulase CBD-CiP fusions were constructed and sequenced.

These JC20-series plasmids were transformed into *S. cerevisae* for expression and testing. After transformation, yeast colonies were grown on selective plates covered with a double filter layer: cellulose acetate filters on top of nitrocellulose. Wild type CiP secreted from yeast JC106 and the stable mutant mCiP842 pass through the cellulose acetate, then binds to the nitrocellulose where it can be visualized using diaminobenzidine (DAB) and H_2O_2. The cellulose acetate filter does not bind any wild-type or mCiP842 peroxidase. In contrast, the N-terminal CBD-CiP fusions encoded by plasmids JC20A, and JC20D are all detectable on both filters using the DAB assay, indicating that the fusion proteins have both peroxidase and cellulose-binding activities. In all cases the peroxidase activity bound to the cellulose acetate filter remains bound even after washing extensively with buffer at pH 7. The activity bound to the lower nitrocellulose filter suggests that binding of the CBD-CiP may be incomplete, or the cellulose filter gets saturated, allowing some of the fusion protein to pass through to the lower filter, or that some percentage of the fusion protein gets truncated to include only the peroxidase domain.

EXAMPLE 2

<u>Enzymatic bleaching of denim (using CiP-CBD or mCiP842-CBD and the enhancer 10-phenothiazine propionic acid)</u>

Denim: Dakota 14 oz. pure indigo denim (standard fabric from Swift, "raw" denim i.e. not stone washed). The denim (75x100 cm) was sewn into "legs" (denim cylinders) weighing approximately 350-375 g each (not stone washed). The denim was then stone washed in de-mineralised water using Aquazym 120 L and Denimax T (obtainable from Novo Nordisk A/S). No carbonate inactivation was made after the stone washing.

Enzyme: CiP-CBD (produced as described in Ex. 1):

approximately 30000 PODU/ml.
mCiP842-CBD, (produced as described in Ex. 1):
approximately 37000 PODU/ml.

Enhancer: PPT (10-phenothiazine propionic acid)
(PPT was produced as described in WO 94/12621 p. 28-29)

H_2O_2: Perhydrol®, 30% (Merck)

Buffer: 0.75 g/l (approximately 5.5 mM) KH_2PO_4 pH 6.5

Equipment: Atlas LP-2 launder-ometer equipped with 1200 ml total volume stainless steel beakers.
All trials were made using 240 ml buffer and 2x12 g denim.
Minolta Chroma Meter CR 300 was used to quantify the bleaching.

Results: ΔL* was measured under the following conditions:
240 ml 5.5 mM phosphate buffer pH 6.5,
2x12 g denim (denim:liquor ratio=1:10),
CiP-CBD: 2 µg enzyme protein per g denim, or
mCiP842-CBD: 3.4 µg enzyme protein per g denim,
1 mM H_2O_2,
2.1 µmole PPT per g denim,
30°C,
30 minutes:

Using CiP-CBD: ΔL*=6.2
Using mCiP842-CBD: ΔL*=5.6.

EXAMPLE 3

<u>Enzymatic bleaching of denim (using CiP-CBD or mCiP842-CBD and the enhancer methylsyringate)</u>

Example 2 was repeated with methylsyringate instead

of 10-phenothiazine propionic acid as enhancing agent.

The following conditions were used:

240 ml 5.5 mM phosphate buffer pH 6.0,

2x12 g denim (denim:liquor ratio=1:10),

CiP-CBD: 3 µg enzyme protein per g denim, or

mCiP842-CBD: 5.1 µg enzyme protein per g denim,

2 mM H_2O_2,

2.9 µmole methylsyringate per g denim,

50°C,

30 minutes.

Results:

ΔL* when using CiP-CBD: 7.2.

ΔL* when using mCiP842-CBD: 9.0.

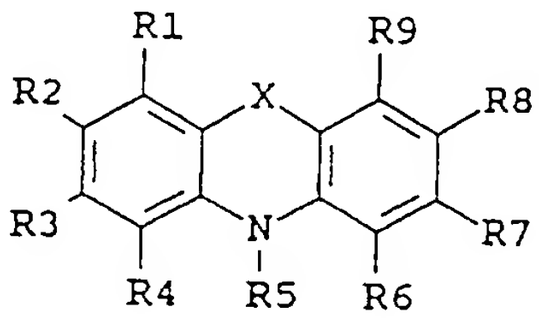
CLAIMS

1. A process for providing a bleached look in the colour density of the surface of dyed fabric, the process comprising contacting, in an aqueous medium, a dyed fabric with

a) a hybrid enzyme comprising a phenol oxidizing enzyme fused to an amino acid sequence having a cellulose-binding domain, and

b) a hydrogen peroxide source when the phenol oxidizing enzyme is a peroxidase.

2. A process according to claim 1, in which the aqueous medium additionally comprises an enhancing agent of the following formula:



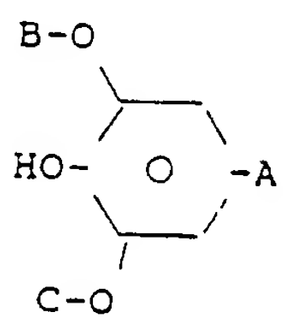
in which formula X represents (-O-) or (-S-), and the substituent groups R^1-R^9, which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, aryl-C_1-C_5-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group R^{10}; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups R^{10}; and which C_1-C_{14}-alkyl, C_1-C_5-alkoxy, carbonyl-C_1-C_5-alkyl, and aryl-C_1-C_5-alkyl groups may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups R^{10};

which substituent group R^{10} represents any of the

following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidino, C_1-C_5-alkyl, C_1-C_5-alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy, C_1-C_5-alkyl, C_1-C_5-alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which C_1-C_5-alkyl, and C_1-C_5-alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl;

or in which general formula two of the substituent groups R^1-R^9 may together form a group -B-, in which B represents any of the following the groups: $(-CHR^{10}-N=N-)$, $(-CH=CH-)_n$, $(-CH=N-)_n$ or $(-N=CR^{10}-NR^{11}-)$, in which groups n represents an integer of from 1 to 3, R^{10} is a substituent group as defined above and R^{11} is defined as R^{10}.

3. A process according to claim 1, in which the aqueous medium additionally comprises an enhancing agent of the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, $-SO_2$-E, -N-XY, and $-N^+$-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C_1-C_{16} alkyl, preferably a C_1-C_8 alkyl, which alkyl may be

saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m-1}; $1 \leq m \leq 5$.

4. A process according to any of claims 1-3, wherein the fabric is dyed with a vat dye such as indigo or thioindigo.

5. A process according to any of claims 1-3, wherein the fabric is a cellulosic fabric or a mixture of cellulosic fibres or a mixture of cellulosic fibres and synthetic fibres.

6. A process according to claim 5, wherein the fabric is denim, preferably denim dyed with indigo or thioindigo.

7. A process according to any of claims 1-3, in which the phenol oxidizing enzyme is a peroxidase.

8. A process according to claim 7, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus or C. macrorhizus, or from Bacillus, e.g. B. pumilus, or Myxococcus, e.g. M. virescens.

9. A method according to any of claims 1-3, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor, e.g. perborate or percarbonate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate, or a peroxycarboxylic acid or a salt thereof.

10. A method according to claim 9, wherein the aqueous medium contains H_2O_2 or a precursor for H_2O_2 in a concentration corresponding to 0.001-25 mM H_2O_2.

11. A process according to any of claims 1-3, in

which the phenol oxidizing enzyme is a laccase or a laccase related enzyme.

12. A process according to claim 11, wherein the laccase is derived from Trametes, e.g., Trametes villosa, or Myceliophthora, e.g., Myceliophthora thermophila, or Coprinus, e.g., Coprinus cinereus.

13. A process according to any of claims 1-3, wherein the concentration of the hybrid enzyme corresponds to 0.001-10000 µg of hybrid enzyme protein per g of fabric.

14. A process according to claim 2, wherein the enhancing agent belongs to the group consisting of phenoxazine-10-propionic acid, phenoxazine-10-hydroxyethyl, phenothiazine-10-ethyl-4-carboxy, phenothiazine-10-propionic acid, promazine hydrochloride and phenothiazine-10-ethylalcohol.

15. A process according to claim 3, wherein the enhancing agent belongs to the group consisting of aceto-syringone, methylsyringate, ethylsyringate, propylsyringate, butylsyringate, hexylsyringate, and octylsyringate.

16. A process according to claim 2 or to claim 3, wherein the enhancing agent in the aqueous medium is present in concentrations of from 0.005 to 1000 µmole per g of fabric.

17. A process according to any of claims 1-3 resulting in reduced strength loss of fabric compared to conventional bleaching processes using e.g. hypochlorite.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00165

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C11D 3/386, D06L 3/02, D06M 16/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C11D, D06L, D06M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9000609 A1 (THE UNIVERISITY OF BRITISH COLUMBIA), 25 January 1990 (25.01.90) --	1-17
A	WO 9218683 A1 (NOVO NORDISK A/S), 29 October 1992 (29.10.92) --	1-17
A	WO 9407998 A1 (NOVO NORDISK A/S), 14 April 1994 (14.04.94) --	1-17
A	WO 9412621 A1 (NOVO NORDISK), 9 June 1994 (09.06.94) --	1-17

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 1997	08.07.97
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Anna Sjölund Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00165

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9623928 A1 (GENENCOR INTERNATIONAL, INC.), 8 August 1996 (08.08.96), page 20, line 26 - line 28 -- --------	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/06/97

International application No.
PCT/DK 97/00165

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9000609 A1	25/01/90	AT 142699 T	15/09/96
		CA 1335182 A	11/04/95
		DE 68927161 D,T	30/01/97
		EP 0381719 A,B	16/08/90
		SE 0381719 T3	
		JP 2528721 B	28/08/96
		JP 3501261 T	22/03/91
		US 5137819 A	11/08/92
		US 5202247 A	13/04/93
		US 5340731 A	23/08/94
WO 9218683 A1	29/10/92	NONE	
WO 9407998 A1	14/04/94	EP 0663950 A	26/07/95
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		JP 8501692 T	27/02/96
WO 9412621 A1	09/06/94	CA 2150562 A	09/06/94
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		CA 2150563 A	09/06/94
		EP 0677102 A	18/10/95
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		JP 8503371 T	16/04/96
		WO 9412620 A	09/06/94
WO 9623928 A1	08/08/96	AU 4902696 A	21/08/96

Form PCT/ISA/210 (patent family annex) (July 1992)